J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 779–783

Effects of β-Lipotropin, β-Endorphin, γ_2-Melanotropin and Corticotropin on Steroid Production by Isolated Human Adrenocortical Cells[1])

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(Received March 27/August 20, 1987)

Summary: Normal human adrenocortical cells (from multi-organ donors) were incubated with corticotropin (tetracosactide), highly purified human β-lipotropin, synthetic human β-lipotropin, γ_2-melanocyte stimulating hormone and β-endorphin. Corticotropin stimulated cortisol, aldosterone and 18-hydroxycorticosterone production starting at 10^{-12}–10^{-11} mol/l in normal adrenocortical cells. Purified human β-lipotropin also stimulated steroidogenesis but 100–1000fold higher concentrations of the peptide were needed. In contrast, synthetic human β-lipotropin was without any effect on steroid production up to concentrations of 10^{-7} mol/l. Synthetic β-lipotropin (5×10^{-10} mol/l) did not significantly change the dose-response curve for corticotropin (10^{-13} mol/l–10^{-9} mol/l) versus the three steroids measured. γ_2-Melanotropin and β-endorphin (10^{-6} mol/l) stimulated the secretion of cortisol, but not of aldosterone.

Since synthetic human β-lipotropin has no effect on human adrenocortical cells, the purified β-lipotropin must be contaminated with traces of corticotropin. Since pathologically elevated levels of proopiomelanocortin-derived peptides will rarely exceed plasma concentrations of 10^{-10} mol/l, our findings in vitro do not support a physiological or pathophysiological role of the peptides examined in the regulation of adrenal steroid secretion.

Introduction

Some experimental data support a role of pituitary factors other than corticotropin in aldosterone regulation, especially during sodium depletion (1, 2). Stimulatory effects of proopiomelanocortin-derived peptides on isolated rat adrenal cells have recently been reported:

Synthetic ovine β-lipotropin (3) and extracted ovine and human β-lipotropin (4) were shown to stimulate aldosterone secretion. *Shanker & Sharma* (5) described a stimulatory effect of synthetic β-endorphin on aldosterone secretion which could not be confirmed by *Matsuoka* et al. (3). Synthetic Lys-γ_3-melanotropin was found to enhance the corticosterone-stimulating effect of corticotropin (6). *Al Dujaili* et al. (7) found that human pro-γ-melanotropin acts synergistically with corticotropin in stimulating corticosterone and aldosterone production by superfused human and rat adrenal cells. Also synthetic β-melanotropin (3) stimulated aldosterone secretion in rat adrenal cells.

[1]) Supported by grants of the Deutsche Forschungsgemeinschaft (Oe47/8–1) and the National Institute of Health (GM-2907).
Presented in part to the 29th annual meeting of the German Endocrine Society, Göttingen, February 20–23, 1985.
Presented in part to the 12th meeting of the Int. Study Group for Steroid Hormones, Rome, December 2–4, 1985.

Reports on effects of proopiomelanocortin-derived peptides on human adrenal cells are scanty. *Al Dujaili* et al. (7) described aldosterone and cortisol stimulating activity of pro-γ-melanotropin on human adrenal cells. *Lis* et al. (8) reported on aldosterone stimulation by γ_3-melanotropin and the 16 K N-terminal sequence of proopiomelanocortin in aldosteronoma cells. *Pham-Huu-Trung* et al. (9) on the other hand, demonstrated an aldosterone-stimulating effect of extracted human β-lipotropin, extracted N-terminal proopiomelanocortin-peptide and synthetic γ_3-melanotropin in normal human adrenal cells and in aldosteronoma cells.

In the study presented here, we investigated steroidogenic effects of synthetic β-lipotropin, γ_2-melanotropin and β-endorphin in comparison with those of corticotropin on normal human adrenocortical cells. To enable comparison with the above-mentioned literature, we investigated the steroidogenic effect of purified human β-lipotropin as well.

Materials and Methods

Human β-lipotropin was isolated and purified, as published previously, by carboxymethylcellulose chromatography and gel filtration, and the homogeneity was tested by paper and disc electrophoresis, and NH_2-terminal analysis (10). Human β-lipotropin was synthesized as published elsewhere (11). Synthetic human (*D*-Ala^2)β-endorphin and γ_2-melanotropin were purchased from Peninsula Lab., California, USA. Homogeneity was tested by high performance liquid chromatography (HPLC), thin layer chromatography (TLC), amino acid analysis and high voltage electrophoresis.

β^{1-24}-Corticotropin (tetracosactide) was purchased from CIBA (Synacthen®). Human adrenal glands were obtained from 10 multi-organ-donors. Immediately after the removal, the glands were placed into cold (4 °C) saline (9 g/l NaCl). As soon as possible, not later than 90 min after the end of blood perfusion, adrenal glands were cut into slices and the outer, subcapsular cell layers were obtained by tangential section of the material for cell preparations. In 6 adrenal cell preparations, the steroid stimulating effect of corticotropin, purified human β-lipotropin and of synthetic γ_2-melanotropin and β-endorphin was investigated (fig. 1). Since extracted β-lipotropin stimulated cortisol secretion in 100—1000 fold higher concentrations than corticotropin, the suspicion arose that the peptide may be contaminated with traces of corticotropin. Therefore, we investigated the effect of synthetic human β-lipotropin alone and in conjunction with corticotropin in 4 further experiments with normal human adrenocortical cells (fig. 2). The detailed procedure of cell preparation and incubation in this laboratory has been described in detail by *Belmega* et al. (12). About 2×10^5 cells (about 90% viable according to trypan-blue exclusion) were incubated in 2 ml medium for 2 hours in a metabolic shaker (Warburg Apparat, type 585G, Braun Melsungen, FRG) under a stream of 95% O_2 and 5% CO_2. The controls were always incubated in duplicate or triplicate. All incubations with corticotropin and with synthetic human β-lipotropin were also carried out in duplicate. Incubations in one vessel only were performed when the effects of purified human β-lipotropin, γ_2-melanotropin and β-endorphin were tested, because the number of incubation vessels in the metabolic shaker was limited to 28 and the supply of human β-lipotropin was small. After the incubation, the decanted cell suspensions were centrifuged at 4000 *g*, and the supernatants were stored at −20 °C until steroid assays were performed.

Cortisol, aldosterone and 18-hydroxycorticosterone were quantified according to *Schöneshöfer* et al. (13). In brief, tritiated probes of the three steroids were added to the samples, which were then twice extracted with 5 ml dichloromethane. This was followed by paper chromatography, localization of the steroids on the paper by tritium scanning and elution with 5 ml methanol. The dried eluates were dissolved in the radioimmunoassay (RIA)-buffer and specific antibodies were used for quantification by RIA. The aldosterone antibody was given to us by the National Institute of Health, Bethesda, Maryland, USA. The cortisol antibody is a gift of Dr. *Vecsei*, Heidelberg, Germany, and the antibody directed against 18-hydroxycorticosterone was prepared in our laboratory (14). The intraassay coefficients of variation for the determination of cortisol, aldosterone and 18-hydroxycorticosterone were 5.1%, 11.0% and 6.0% respectively, the interassay coefficients of variation 20.4%, 23.5% and 18.8% respectively. The sensitivity of the assays (90% B_o) was 75 pmol/l cortisol, 85 pmol/l aldosterone and 104 pmol/l 18-hydroxycorticosterone. In most experiments, cortisol concentrations were measured without paper chromatography, after comparative measurements had shown that cortisol levels following chromatography were only 10% lower, and the coefficient of correlation between the two methods was 0.83.

Results

Since the adrenals from cadaver kidney donors could not be obtained under standardized conditions, basal secretory rates and steroid stimulation by the peptides investigated exhibited a large variability. Basal steroid secretions are shown in table 1, while the results in the figures are expressed as relative changes over control levels. The significance of differences between controls and stimulated samples was calculated using the paired t-test.

Tab. 1. Unstimulated basal steroid production rates (ng/10^5 cells per 2 hours) of 10 adrenals from different donors.

	Range	$\bar{x}$	SEM
Cortisol	28 —415	124	42.6
Aldosterone	0.16— 9.78	2.5	1.23
18-Hydroxycorticosterone	0.14— 23.2	3.57	2.65

Figure 1 shows the results of six experiments with adrenocortical cell incubations, where dose-response curves with purified human β-lipotropin, synthetic β-endorphin and synthetic γ_2-melanotropin were compared with a high and low dose of corticotropin. Corticotropin (10^{-9} mol/l) stimulated cortisol five- to sixfold, aldosterone about twofold and 18-hydroxycorticosterone about threefold. β-endorphin and γ_2-melanotropin (10^{-6} mol/l) stimulated cortisol significantly, but not the zona glomerulosa steroids. At 10^{-7}—10^{-6} mol/l extracted human β-lipotropin

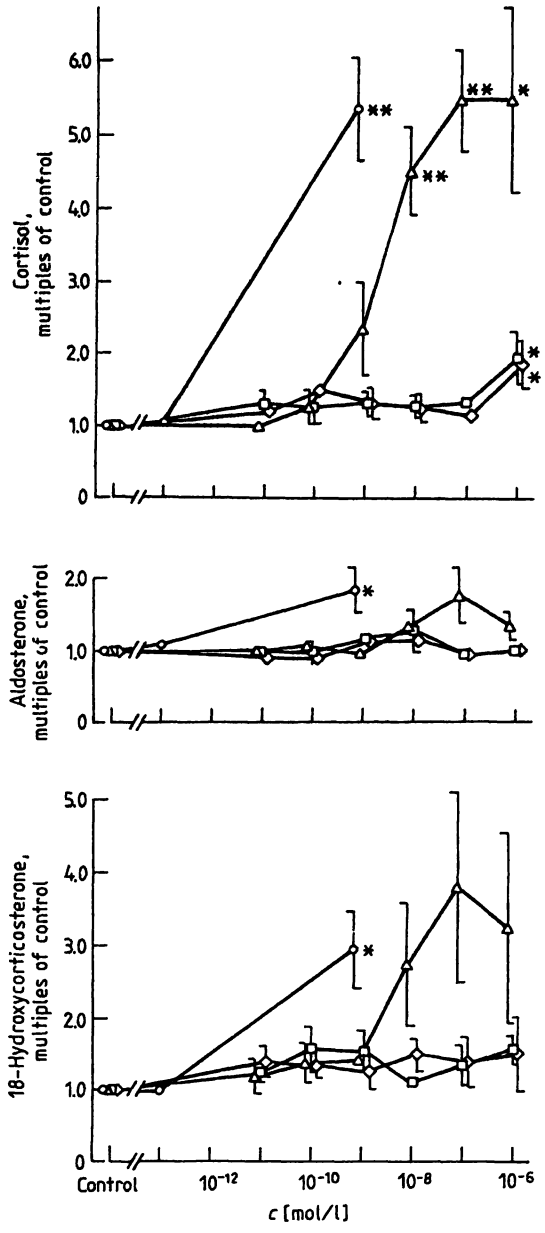

Fig. 1. Effects of corticotropin 1—24 (○), highly purified human β-lipotropin (△), synthetic β-endorphin (□), and γ_2-melanotropin (◇) in six experiments with adrenocortical cells from cadaver kidney donors. Production rates of cortisol, aldosterone and 18-hydroxycorticosterone in unstimulated control vials (duplicate or triplicate) are defined as 1.0. The increase (multiples ± SEM) is shown.
* = $p \leqslant 0.05$, ** = $p \leqslant 0.01$ (*Student*'s t-test) compared with control incubations.

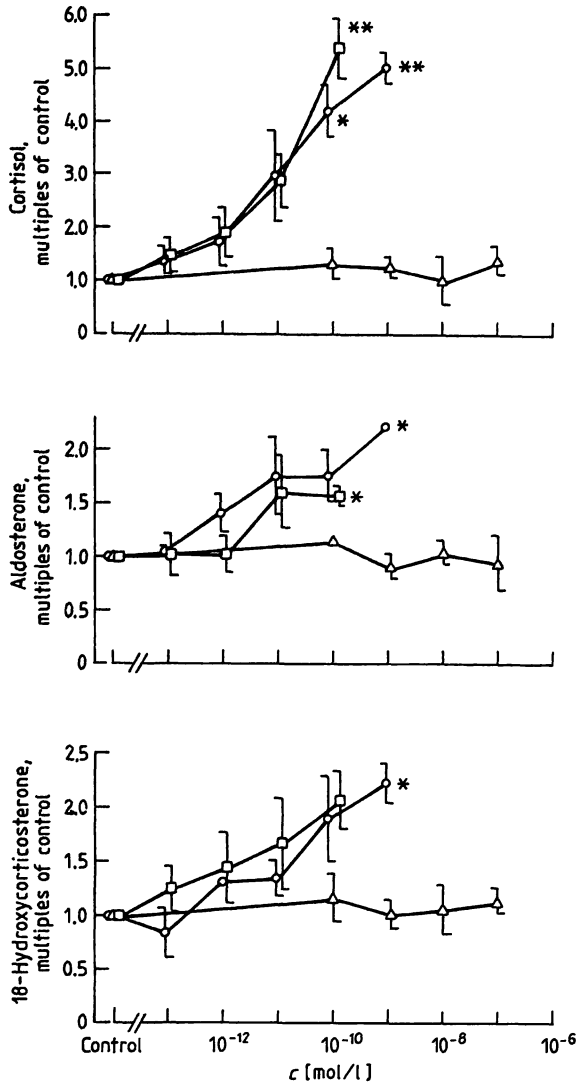

Fig. 2. Effects of corticotropin 1—24 of (○), synthetic human β-lipotropin (△) and $5 \cdot 10^{-10}$ mol/l synthetic human β-lipotropin together with rising concentrations of corticotropin 1—24 (□) on production of cortisol, aldosterone and 18-hydroxycorticosterone in four preparations of adrenocortical cells from cadaver kidney donors. Mean changes (multiples ± SEM) of steroid production over control levels (1.0) are shown.
* = $p \leqslant 0.05$, ** = $p \leqslant 0.01$ (*Student*'s t-test) compared with control incubations.

stimulated all three steroids to the same extent as 10^{-9} mol/l corticotropin, but changes in aldosterone and 18-hydroxycorticosterone were not significantly different from the controls ($p > 0.05$) due to larger variability.

Figure 2 demonstrates the results of four experiments with adrenal cells, where dose-response curves with corticotropin and *synthetic* human β-lipotropin were set up. Synthetic human β-lipotropin had no effect whatsoever on steroid production up to concentrations of 10^{-7} mol/l. Corticotropin started to stimulate cortisol at 10^{-12} mol/l. The cortisol rise at 10^{-9} mol/l corticotropin was probably close to the maximum. The stimulation curves of aldosterone and 18-hydroxycorticosterone were flatter with the threshold concentration of corticotropin at about 10^{-11} mol/l. When 5×10^{-10} mol/l synthetic human β-lipotropin was added to all incubates, the dose response curve (corticotropin vs steroid production) was not significantly modified, although small influences of β-lipotropin may have remained undetected because of the large variability of the individual cell preparations.

Discussion

The basal secretory rates of adrenocortical cells are similar to those reported recently by *Belmega* at al. (12). However, the cell preparations used in the present study were less sensitive to corticotropin. Basal and stimulated levels of 18-hydroxycorticosterone were about two- to threefold higher than those of aldosterone. A similar ratio between the two zona glomerulosa products is also found in plasma (15).

While cortisol secretion is markedly stimulated by corticotropin in isolated human adrenocortical cells, the stimulation of aldosterone and 18-hydroxycorticosterone is less impressive than that found in vivo. This may be due to functional changes during glomerulosa cell preparation (16). Isolated cells from the zona glomerulosa of *rat* adrenals are easier to handle, and the results are probably more reproducible than those with human zona glomerulosa cell preparations. However, it is doubtful whether findings in the rat are always transferable to human biology.

We found that purified human β-lipotropin stimulated cortisol and the zona glomerulosa steroids to the same extent as corticotropin, although at 100—1000fold higher concentrations. A similar observation was made by *Washburn* et al. (4) using rat adrenocortical cells. Synthetic β-lipotropin (ovine) was inactive in the rat (4), just as human synthetic β-lipotropin was inactive in our human adrenocortical cell preparations. The discrepancy between the effects of extracted human β-lipotropin and synthetic human β-lipotropin may be due to small amounts of corticotropin present in the extracted peptide preparation. This may also explain the stimulatory effect of extracted human β-lipotropin in aldosteronoma cells reported by *Pham-Huu-Trung* et al. (9).

According to *Smith* et al. (17) β-lipotropin concentrations in normal human plasma are about 10^{-11} mol/l. Following insulin-induced hypoglycaemia, β-lipotropin levels rise in the same way as those of corticotropin. In untreated patients with *Addison*'s disease, β-lipotropin levels may rise up to fiftyfold above normal. If the activity of extracted human β-lipotropin at 10^{-9} mol/l (threshold dose for cortisol and 18-hydroxycorticosterone) was not due to contamination with corticotropin, extremely high pathological plasma concentrations of β-lipotropin might exert some biological effect on the adrenal gland, as for example in patients with *Cushing*'s syndrome due to ectopic corticotropin (and β-lipotropin) secretion. A physiological contribution of β-lipotropin to the regulation of adrenocortical function, however, is unlikely. Our second experiment, where cells were incubated with corticotropin plus β-lipotropin, were carried out with the synthetic human peptide which had no steroidogenic potency of its own. No modification of the effect of corticotropin on the three steroids was found.

γ_2-Melanotropin as well as β-endorphin had a small stimulatory effect on cortisol secretion at the very high concentration of 10^{-6} mol/l, while the zona glomerulosa steroids remained unchanged. This concentration seems to be far outside the physiological range: *Hope* et al. (18) found the molar concentration of the immunoreactive N-terminal portion of proopiomelanocortin in human plasma to be about two- to threefold higher than those of corticotropin. Even in plasmas from patients with greatly elevated corticotropin concentrations (ectopic corticotropin syndrome, *Addison*'s disease), immunoreactive N-terminal portion of proopiomelanocortin was below 10^{-9} mol/l. Plasma concentrations of β-endorphin in the human are of the same order of magnitude as those of corticotropin (17). A physiological role of γ-melanotropin peptides and/or β-endorphin in the function of the adrenal gland is therefore unlikely, if our in vitro results are taken into account. However, *Al-Dujaili* et al. (7) observed a potentiating effect of rather low concentrations of pro-γ-melanotropin on aldosterone and corticosterone secretion by isolated superfused human and rat adrenocortical cells. Pro-γ-melanotropin itself did not stimulate steroid secretion. *Farese* et al. (6) also described a synergistic effect between corticotropin and γ_3-melanotropin (corresponding to γ_2-melanotropin with 15 additional C-terminal amino acids) in corticosterone-producing zona fasciculata cells of rats.

Recently, an in vivo stimulating effect of β-endorphin on aldosterone secretion was demonstrated in man (19). An infusion rate of 1 μg/kg · min stimulated plasma aldosterone and renin activity and led to a fall in plasma corticotropin and cortisol. The latter effect may be due to inhibition of corticotropin secretion by a feedback mechanism at the level of the hypothalamus, while the increase in aldosterone may be mediated by the renin-angiotensin system. In contrast, *Kem* et al. (20) found no effect on plasma aldosterone or plasma cortisol with bolus administrations of up to 15 mg β-endorphin in four normal human subjects. Our own in vitro results with β-endorphin speak against a direct effect of this peptide on the adrenal cortex, thereby confirming the findings of *Matsuoka* et al. (3) in rat adrenal cell preparations.

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